Pharmacological strategies to interfere with proinflammatory signal transduction in endothelial cells

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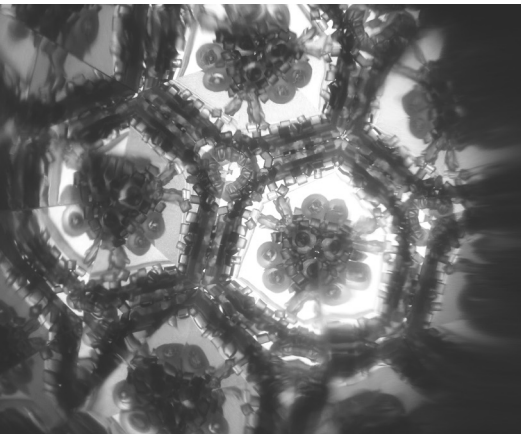

Chapter 8

General discussion and perspectives

Inflammation is a naturally occurring process that exists as a first response of the immune system to infection, and as such it is considered a "good" process necessary for maintenance of body homeostasis. However, in the case of chronic inflammatory diseases like rheumatoid arthritis and glomerulonephritis, ongoing leukocyte activation and recruitment often leads to severe tissue disruption and loss of organ function. Many studies hypothesized, and demonstrated, that control of inflammation by inhibition of (immune) cell activation and leukocyte influx resulted in improved disease outcome. Different therapies have been proposed that interfere with leukocyte recruitment at different levels. These include antibodies or small antibody fragments to neutralize proinflammatory cytokines, or functionally inhibit adhesion molecules on leukocytes and endothelial cells (1). Therapies that entered the market are among others directed to neutralize tumor necrosis factor (TNF)α activity in rheumatoid arthritis and Crohn's disease (Infliximab, Remicade ®) and α_4-integrin binding to very late antigen (VLA)-4 (Natalizumab, Tysabri ®). Other "magic bullet" therapies, although successful in preclinical studies, showed limited outcome in clinical trials. A likely explanation for the observed lack of effects is that blocking one specific molecule involved in leukocyte recruitment can lead to functional replacement by alternative molecules. Moreover, since different leukocyte subsets employ different combinations of adhesion molecules, cytokines and chemokines for their extravasation and activation, inhibition of expression of only one of these molecules may not significantly alter the extent of leukocyte recruitment (for a review, see (2)).

A growth in understanding of the functional and molecular control of intracellular signal transduction during cell activation has led to a shift in drug development from antibodies and peptides that block specific molecules that control leukocyte – endothelial cell interaction to small chemical entities that affect inflammatory cell activation. In this thesis we studied the effects of selective interference with two prime proinflammatory intracellular signaling pathways nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) in endothelial cells employing low molecular weight chemical inhibitors and a gene therapy vector. NF-κB signal transduction blockade resulted in strong inhibition of adhesion molecule gene expression and only limited inhibition of cytokines and chemokines, while blockade of p38 MAPK signaling resulted in moderate (E-selectin) or no (vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1)) effect on adhesion molecules while significant inhibition of cytokine and chemokine expression was observed. These experiments confirmed a major role for both NF-κB and p38 MAPK pathways in regulation of gene expression in endothelial cells as part of the inflammatory response induced by cytokines.

We hypothesized that blocking both pathways simultaneously would significantly enhance the pharmacological effect, and experimentally supported this hypothesis in our *in vitro* model system employing chemical inhibitors. Combining multiple drugs in a therapeutic regimen is a feasible approach which is at present under investigation in our laboratory. One potential drawback of freely administered (combinations of these) drugs may be the induction of toxicity in normal tissues, as both NF-κB and p38 MAPK are considered also of importance for normal cell homeostasis (3;4). To specifically direct

such a combination therapy to the endothelium in inflammatory sites, the drugs could be included in immunoliposomes harnessed with antibodies specifically recognizing the diseased endothelial cells. These targeted drug delivery systems require the candidate drugs to have a hydrophilic character, to prevent the drugs from redistribution prior to reaching the target upon systemic administration. Additionally, the candidate drugs should exert a proper pharmacological effect at concentrations that can be achieved by targeted drug delivery. A number of drugs used in our studies were effective at low micromolar concentrations. However, all compounds employed were hydrophobic by nature, which appeared to be the major limiting factor for application in a liposome-based drug delivery formulation. In general, new chemical entities in the drug development pipeline are selected for further study based on their pharmacological effects in cell systems. Since this quality is intricately associated with the ability to cross biological membranes (*i.e.,* with a hydrophobic nature), many of the new kinase inhibitors that were excluded from further clinical testing due to unacceptable toxicity and as such are interesting candidates for targeted delivery, are not qualified for immunoliposomal delivery unless chemically modified.

Instead of an immunoliposome-based approach, we investigated in Chapters 5 and 6 whether adenoviral gene therapy vectors could be considered for selective delivery of a therapeutic transgene that interferes with intracellular signaling. Recombinant adenoviruses have a number of important characteristics that make them useful as gene transfer tools for application in gene therapy (5). They are easy to propagate and can transduce a wide range of cells with high efficiency. Moreover, their genomes can accommodate relatively large fragments of DNA. The viral genome is maintained epichromosomally through numerous replication cycles and it is believed to not to affect host genes (6). These advantageous features have led to a rapid development of the field of recombinant adenoviruses for therapeutic application. Yet, despite promising results in the laboratory, these adenoviruses have not been extremely successful in clinical settings. One of the major reasons is the unrestricted distribution of adenoviral gene transfer to nontarget tissues, with markedly reduced therapeutic effects and vector-induced immunogenic toxicities. In our research we aimed to circumvent these problems by introducing activated endothelial cell-specific antibodies combined with polyethylene glycol (PEG) modification of the adenovirus capsid. This resulted in alteration of the natural viral tropism and selective transfer of the transgene into activated endothelial cells expressing E-selectin or VCAM-1. In nature, adenovirus infection occurs through the interaction of the viral fiber protein and its primary cell surface receptor, Coxsackie and adenovirus receptor (CAR) (7). We redirected this adenovirus interaction to alternative cell surface receptors which are inflammation induced. E-selectin is especially attractive due to its high and transient expression restricted to activated endothelial cells in microvasculature engaged in inflammatory processes. Another inflammation associated epitope, VCAM-1, was recently described as a potential target for specific drug delivery to activated endothelial cells (8). One of the drawbacks of E-selectin as a target epitope when considering clinical application may be its transient expression – when the patient presents in the clinic with (recurrent) disease, this adhesion molecule may already have been downregulated. In contrast,

VCAM-1 is often expressed at a later moment in the inflammatory process, and its expression is generally believed to be more prolonged. Yet, employing VCAM-1 as a target may have the intrinsic weakness that it is constitutively expressed *e.g.*, in the kidney peritubular endothelium (Chapter 4), which may presents as a potential site for unwanted side effects of the targeted therapeutic.

In our endothelium-directed gene therapy strategy we employed dominant-negative inhibitor κB (dnIκB) as a transgene, which selectively blocked NF-κB signaling in activated endothelial cells. NF-κB signal transduction induces the expression of multiple downstream effector molecules including cell adhesion molecules (P- and E-selectin, VCAM-1, ICAM-1), cytokines, and chemokines. These molecules serve as guidance for recruitment of different subsets of immune cells during inflammation. A dose of 10^4 viral particles per cell nearly abolished NF-κB signaling and consequently the expression of the inflammatory molecules, thereby confirming the potential of dnIκB inhibitory protein as an anti-inflammatory therapeutic protein with broad effector profile. We did observe anti-inflammatory effects of the retargeted dnIκB adenovirus in a mouse model of glomerulonephritis (Chapter 6), yet some issues remain unsolved. For example, the number of viral particles delivered per cell *in vivo* is unknown. Moreover, we do not know whether a dose of viral particle per cell needed in glomerular endothelium to silence the NF-κB pathway is similar to the dose needed in human umbilical vein endothelial cells (HUVEC).

Of interest is the observation that, when combining virus targeted NF-κB inhibition with chemical drug based inhibition of p38 MAPK (Fig. 1), a complete blockade of gene expression could not be achieved, similar to the observations reported in Chapter 3. In addition, in Chapter 4, we observed that blockade of NF-κB in TNFα activated HUVEC resulted in an increase in p38 MAPK activity (Chapter 4, Fig. 4). This data imply that in endothelial cells signaling pathways additional to NF-κB and p38 MAPK prevail when others are inhibited. To elucidate the intracellular downstream events responsible for these observations, more detailed investigations on kinase activation profiles during cell activation and drug treatment are necessary. Novel approaches including kinase array assays and progress in proteomics and computer technology need to be employed to learn to understand complexity and kinetics of kinase activities in a cell under normal conditions, and in response to stress and therapeutic intervention.

The endothelium on one hand is considered as an integrated system covering all blood vessels that traverse the body, while on the other hand, each vascular bed has unique structural and functional properties with consequences for endothelial behavior (Fig. 2). Each tissue contains its own highly specialized microvascular endothelium, which displays organ-specific characteristics. For example, in the brain the tightly connected nonfenestrated continuous endothelium from the blood brain barrier is highly specialized to protect the neural tissue from fluctuations in blood composition. In contrast, the fenestrated endothelium in the glomeruli in the kidney is highly specialized to facilitate the blood filtration process. While knowledge regarding macrovascular endothelial physiology and dysfunction in cardiovascular diseases has increased considerably in the last decades, the molecular complexity of the microvascular endothelium has not

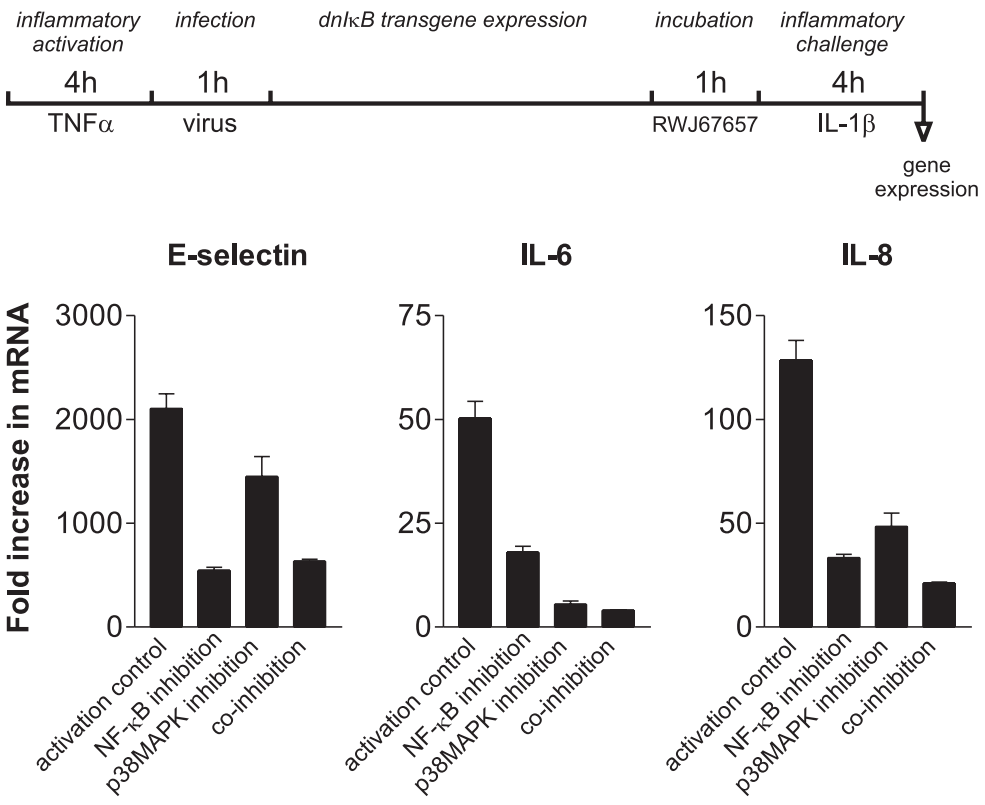

Figure 1. Effect of simultaneous inhibition of NF-κB and p38 MAPK signaling on inflammatory gene expression in HUVEC. TNFα-activated HUVEC were transfected with Ab_{Esel}-PEG-AddnIκB and treated with 1 μM RWJ67657 before activation with interleukin (IL)-1β (scheme represents the experimental protocol). Gene expression was analyzed using quantitative RT-PCR and data were adjusted to the expression of GAPDH.

been extensively studied. Since inflammatory processes and angiogenic remodeling mainly take place in the microvasculature, a detailed understanding of the behavior of these cells in health and disease, and in response to drug treatment will be essential for the development of clinically effective therapeutic regimens. Therefore we set out to investigate the molecular make-up of microvascular endothelial cell subsets within one particular organ. We were able to study endothelium in its *in vivo* context using a new approach in which we combined endothelial cell laser microdissection with quantitative RT-PCR gene expression profiling. This experimental approach is of interest for scientists working in basic (endothelial) biomedicine research, to unravel the *in vivo* mechanisms underlying (vascular) diseases, and in the different expertise fields of drug development. In this thesis research, we applied the microdissection strategy combined with quantitative gene expression analysis to approach different subsets of endothelial cells in the kidney in different (patho)physiological and pharmacological conditions. We showed that in basal conditions subsets of endothelial cells exerted differential patterns of endothelial marker gene expression. For example, the ratio of CD31 :

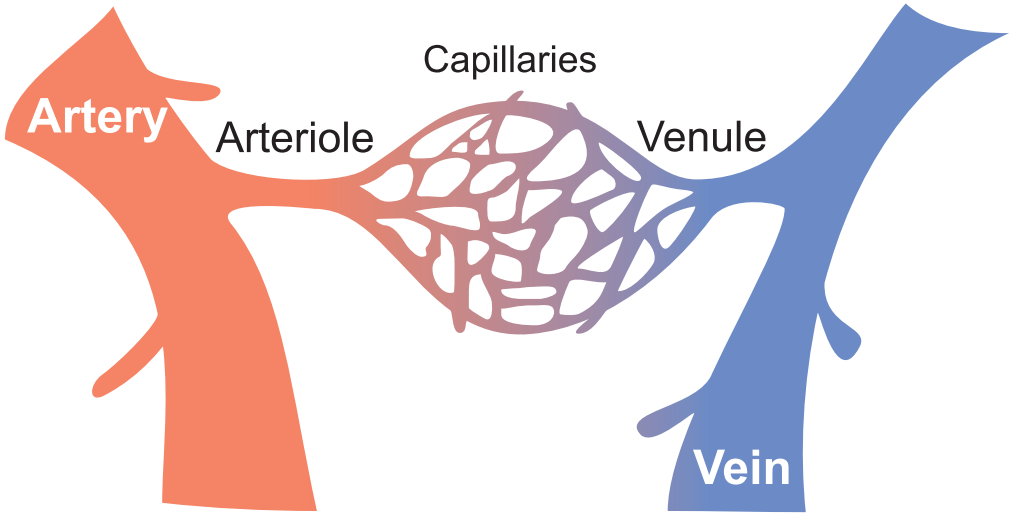

Figure 2. Organization of the vascular tree. Circulating blood travels from arteries through arterioles and capillaries to (post-capillary) venules, from where it enters the veins. All vascular beds are lined by endothelial cells that exert characteristic functions depending on their location in the body.

VE-cadherin was approximately 2, 0.7, and 1 in arterioles, respectively glomerular and venular endothelium. Both genes are involved in maintaining endothelial integrity and in signaling events during inflammatory and angiogenic episodes. Possibly, these differences in gene expression levels, which were substantiated by protein expression using immunohistochemistry, have a functional meaning which is unknown at present. The observation that endothelial marker genes are differentially expressed furthermore has implications for their use as endothelial housekeeping gene/RNA reference in laser microdissection microscopy/quantitative RT-PCR protocols as well as for use in immunohistochemical identification of endothelial subsets prior to dissection.

The biology of different endothelial cell types has been extensively studied in cell culture systems. Although these studies added to our understanding of basic endothelial cell behavior, they miss the actual context of the environment. The endothelial surroundings are essential in maintenance of function in the particular tissues. For example, in glomeruli, podocytes and mesangial cells were both shown to communicate with glomerular endothelium to support the physiological filtration function of the capillaries (9). In inflammation, microvascular endothelial cells become activated by proinflammatory cytokines, of which the local concentrations are determined by the pathophysiological environment. Endothelial cells respond to this activation by quick and well-organized changes in gene expression, with their protein products serving to recruit different subsets of immune cells. We shed some light on the complexity of this system when studying the *in vivo* microvascular reaction to TNFα. Not all vascular beds in the organs expressed the expected combination of selectins and integrin- and immunoglobulin super family adhesion molecules. Possibly, the 2h time frame was too short to mount a complete microvascular reaction, although all inflammatory genes

studied were upregulated at the mRNA level at this time point. It is therefore likely, that immune cell subset recruitment is endorsed by a site restricted control of endothelial gene expression. Moreover, during the inflammatory event, the recruitment of the leukocytes by cell-cell contact with the endothelium can also influence endothelial cell behavior. For example during inflammation in the central nervous system, ICAM-1-mediated signaling in brain endothelial cells is a crucial regulatory step in the process of lymphocyte migration through the blood brain barrier (10). As such, this represents an additional complexity in *in vivo* microvascular behavior which cannot be easily mimicked in *in vitro* culture systems.

In our *in vivo* studies on endothelial cell responses to drug treatment during acute inflammatory activation we observed that the inflammatory genes affected by the treatment did not represent the ones that responded in the isolated endothelial cell system *in vitro*. Among the striking differences was the absence of effects of dexamethasone (DEX) on cytokine-induced adhesion molecule expression in HUVEC, while *in vivo* the drug significantly inhibited the expression of a number of these genes. In contrast, the activator protein (AP)-1/NF-κB inhibitor MOL-294 exhibited tremendous effects on adhesion molecules *in vitro*, while it was devoid of effects *in vivo*. These data demonstrated that the *in vitro* pharmacological activity of the anti-inflammatory drugs were not representative of the effects *in vivo*, where endothelial cells are present in their natural context and experience direct interaction with blood and blood borne entities. Although we studied the effects of drug pretreatment on acute proinflammatory endothelial activation in a relatively short (2h) time frame, it cannot be excluded that the responses *in vivo* have been influenced by effects of the drugs on *e.g.,* leukocytes. Their intricate interaction with the microvascular endothelium during each circulation event may (indirectly) affect endothelial behavior. Irrespective of the reason for the discrepancy observed, the conclusion remains justified that *in vitro* endothelial responses to drugs are not *per se* indicative of their responses *in vivo*. Careful *in vivo* evaluation of the pharmacological effects in diseased cells within an organ is warranted to prevent misinterpretation of data.

It is important to realize that the added therapeutic value of pharmacological interference with activated endothelial cells still needs to be determined. It has not been clearly established yet, whether chronic inflammation will resolve by inhibiting endothelial cell activation only. Research in our laboratory showed that treatment with E-selectin directed immunoliposomes containing DEX strongly reduced glomerular proinflammatory gene expression in mice suffering from glomerulonephritis (11). Moreover, the delivered DEX reduced renal injury as evidenced by a reduction in blood ureum nitrogen levels and decreased glomerular crescent formation. This study not only exemplified the use of innovative techniques to study endothelial vascular bed specific responses to targeted therapy, it also demonstrated one of the first attempts to bridge microvascular gene expression profiles with actual disease outcome.

In summary, one can conclude that pharmacological intervention with microvascular endothelial cell activation has therapeutic potential. However, several issues need further attention, including drug choice in relation to the vascular bed

aimed at, formulation of the drugs, the timing of administration and optimal dose, as well as the choice of (surrogate) biomarker for measuring effects. Knowledge about this will contribute to the development of improved therapies for inflammatory diseases.

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